Immunity
Article

Sensing of Commensal Organisms by the Intracellular Sensor NOD1 Mediates Experimental Pancreatitis

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SUMMARY

The intracellular sensor NOD1 has important host-defense functions relating to a variety of pathogens. Here, we showed that this molecule also participates in the induction of a noninfectious pancreatitis via its response to commensal organisms. Pancreatitis induced by high-dose cerulein (a cholecystokinin receptor agonist) administration depends on NOD1 stimulation by gut microflora. To analyze this NOD1 activity, we induced pancreatitis by simultaneous administration of a low dose of cerulein (that does not itself induce pancreatitis) and FK156, an activator of NOD1 that mimics the effect of gut bacteria that have breached the mucosal barrier. The pancreatitis was dependent on acinar cell production of the chemokine MCP-1 and the intrapancreatic influx of $CCR2^+$ inflammatory cells. Moreover, MCP-1 production involved activation of the transcription factors NF-κB and STAT3, each requiring complementary NOD1 and cerulein signaling. These studies indicate that gut commensals enable noninfectious pancreatic inflammation via NOD1 signaling in pancreatic acinar cells.

INTRODUCTION

Although most episodes of acute pancreatitis are mild, a subpopulation of patients with this condition develops a severe disease with local and extrapancreatic complications (Frossard et al., 2008). Bacterial colonization of the inflamed pancreas is involved in the latter cases and, in fact, infection of necrotic pancreatic tissue is one of the most important causes of mortality in acute pancreatitis (Frossard et al., 2008). It is now generally accepted that such colonization and associated inflammation result from failure of intestinal-barrier function and translocation of intestinal microflora into the splanchnic vascular bed (Frossard et al., 2008; Rychter et al., 2009).

Microbe-associated molecular patterns (MAMPs) derived from the intestinal microflora activate the host innate immune system via pattern-recognition receptors such as Toll-like receptors (TLRs) and nucleotide-binding domain and leucine-rich repeat containing molecules (NLRs) (Akira and Takeda, 2004; Chen et al., 2009; Strober et al., 2006; Werts et al., 2011). Thus, it is probable that activation of TLRs and NLRs is involved in the mechanisms by which bacterial translocation accounts for the development of severe acute pancreatitis. Consistent with this idea, the severity of acute pancreatitis is ameliorated in mice lacking TLR4 (Sharif et al., 2009), and polymorphism in the TLR genes is associated with susceptibility to acute pancreatitis (Gao et al., 2007; Takagi et al., 2009). In addition, NF-κB, a downstream transcription factor of the TLR and NLR signaling pathways (Akira and Takeda, 2004; Strober et al., 2006), plays a critical role in the development of acute pancreatitis (Baumann et al., 2007; Rakonczay et al., 2008; Tando et al., 1999).

Studies have highlighted the role of the NLR family of proteins in the microbial-recognition system that functions in the intestinal milieu (Strober et al., 2006; Chen et al., 2009; Werts et al., 2011). NOD1, which belongs to this family, is of particular interest because it has been shown to play a protective role in infection of the mucosal surface (Strober et al., 2006). NOD1 contains a leucine-rich repeat region that serves as an intracellular sensor of small peptide components derived from bacterial peptidoglycan (PGN). Such recognition leads to NOD1 activation and the production of proinflammatory mediators, either through nuclear translocation of NF-κB or through interferon regulatory factors (IRFs) and type I interferon signaling (Chamaillard et al., 2003; Fritz et al., 2007; Watanabe et al., 2010).

The above properties of NOD1 suggest the possibility that this NLR family member could contribute to the development of noninfectious inflammatory states, particularly if it can be shown that NOD1 responds to gut commensal organisms as well as to pathogenic organisms (Girardin et al., 2003; Kim et al., 2004). Here, we addressed this possibility by defining the role of NOD1 in the development of cholecystokinin receptor (CCKR) agonist-induced acute pancreatitis (cerulein pancreatitis). In

a key initial finding, we showed that administration of high doses of cerulein, a well-established inducer of pancreatitis, requires the presence of gut commensal organisms acting through NOD1 for the development of pancreatic inflammation. This observation led us to develop a model of pancreatitis that would allow us to define the role of NOD1 signaling in pancreatitis. This consisted of the administration of low doses of cerulein that do not in themselves cause pancreatitis and of the administration of NOD1 ligand, which in this case could be shown to mimic the activity of gut commensal bacteria that enter circulation during pancreatitis. Using this model, we showed that NOD1 stimulation facilitates the migration of $CCR2^{+}$ myeloid cells to the pancreas in response to the robust acinar cell production of monocyte chemotactic protein-1 (MCP-1); the latter, in turn, results from cooperative NOD1-cerulein activation of NF-κB and STAT3. Overall, these findings reveal that NOD1 signaling plays a major role in the pathogenesis of cerulein-induced acute pancreatitis via its capacity to respond to commensal gut bacteria.

RESULTS

Bowel Sterilization by Broad-Spectrum Antibiotics Inhibits High-Dose Cerulein Pancreatitis

Repeated administration of high doses of cerulein (50–100 μg/kg), a CCKR agonist, is a well-established inducer of acute murine pancreatitis (Baumann et al., 2007; Sharif et al., 2009). To address the role of commensal organisms in the intestinal microflora in the development of high-dose cerulein pancreatitis, C57BL6 mice were administered a combination of antibiotics (ampicillin [AMP], vancomycin, neomycin, and metronidazole) in the drinking water for 3 weeks, a regimen previously used to achieve bowel sterilization (Fagarasan et al., 2002), and then challenged with repeated systemic intraperitoneal (i.p.) injections of high doses of cerulein (50 μg/kg). We found that mice administered normal drinking water and given seven hourly injections of cerulein developed severe pancreatitis associated with a marked increase in the serum concentrations of pancreatic enzymes, amylase, and lipase; in contrast, mice preadministered drinking water containing antibiotics developed barely detectable pancreatic inflammation and only marginal elevations in the serum concentrations of pancreatic enzymes (Figures 1A and 1B). In complementary studies addressing the effects of a single commensal organism, mice were first administered ampicillin and kanamycin (KM) in the drinking water for 3 weeks and then challenged with high doses of cerulein as before. We observed that, even with this less stringent bowel-sterilization regimen, the mice exhibited only a mild elevation in the serum concentrations of amylase associated with normal pancreatic architecture (Figures S1A and S1B available online). However, high serum amylase concentrations and severe pancreatic inflammation were again observed in mice that were administered AMP- and KM-containing drinking water and underwent periodic oral administration of AMP- and KM-resistant *E. coli* expressing LacZ (ECLACZ) (Yoshida et al., 2001) (Figures S1A and S1B). In contrast, periodic oral administration of ECLACZ alone did not induce a significant elevation of serum amylase levels (Figure S1C) and, in addition, did not enhance elevated serum amylase levels that were caused by administration of cerulein in the absence of AMP and KM in the drinking water (Figure S1D). Thus, ECLACZ used in this study behaved like a commensal organism, with no capacity to induce pancreatitis on its own. Taken together, these data provide strong evidence that commensal organisms in the intestinal microflora are essential for the development of acute pancreatitis caused by high-dose cerulein.

NOD1 Activation Is Necessary for the Development of High-Dose Cerulein Acute Pancreatitis

The above results suggested that an intestinal microbial component recognized by a pattern-recognition receptor is important in the development of high-dose cerulein pancreatitis. To investigate this possibility, we compared serum concentrations of amylase after high-dose cerulein injections, as described above, in mice deficient in TLR2, TLR4, TLR9, or NOD1. Wild-type (WT) mice, TLR2-deficient ($Tlr2^{-/-}$) mice, and $Tlr9^{-/-}$ mice all developed similar increases in the serum concentration of amylase, suggesting that TLR2 and TLR9 are not required for pancreatitis induction (Figure 1C). In contrast, $Tlr4^{-/-}$ mice exhibited a significant decrease in serum amylase as compared to WT mice. This is consistent with a report that TLR4 activation is involved in pancreatitis induction (Sharif et al., 2009). More surprisingly, however, cerulein-induced elevations in serum amylase concentrations were almost completely abrogated in $Nod1^{-/-}$ mice (Figure 1C). These data suggest a critical role for NOD1, which senses a peptide derived from PGN of the intestinal bacteria in the induction of high-dose cerulein pancreatitis.

Low-Dose Cerulein Together with NOD1 Ligand Induces Pancreatitis

We sought an experimental model in which NOD1 ligand administration could be used as a substitute for MAMPs derived from gut bacterial flora so that the effects of NOD1 signaling could be easily isolated from those of cerulein. In searching for such a model we became aware that whereas repeated administration of high doses of cerulein (50–100 μg/kg) induces acute pancreatitis as indicated above, administration of low doses of cerulein does not have this effect, although such doses are still capable of acinar cell signaling (Baumann et al., 2007; Sharif et al., 2009). This suggested that low-dose cerulein does not induce pancreatitis because it does not by itself cause an influx of gut microorganisms sufficient to result in robust acinar cell NOD1 signaling, and thus low-dose cerulein would cause pancreatitis if administered with NOD1 ligand. To test this possibility, we administered FK156 (NOD1 ligand) or muramyl dipeptide (MDP, NOD2 ligand) (each at a dose of 200 μg i.p.) to C57BL6 mice, 2 hr prior to initiation of a low-dose cerulein regimen consisting of three hourly injections of a dose of cerulein (20 μg/kg i.p.) that does not induce pancreatitis when administered alone (Figures 2A and 2B). We found that serum concentrations of amylase and lipase were markedly elevated in mice treated with FK156 plus low-dose cerulein, but not in mice treated with MDP plus low-dose cerulein or in mice treated with either ligand or cerulein alone (Figure 2B). In addition, pancreatic edema, necrosis, and infiltration of immune cells were observed in mice treated with FK156 plus cerulein, but not under the other conditions (Figures 2C and 2D). Finally, we showed that comparable amounts of serum amylase were obtained when mice were

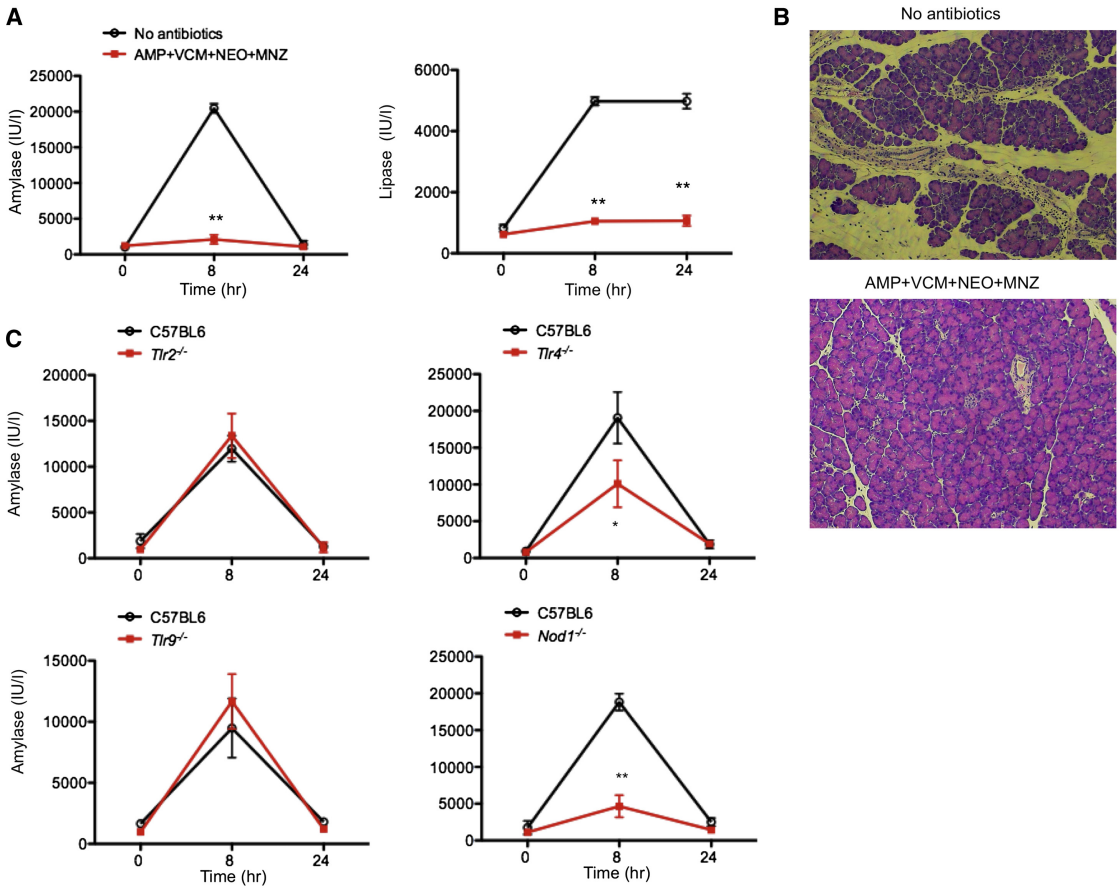


Figure 1. Bowel Sterilization by a Broad Range of Antibiotics Inhibits Development of Cerulein-Induced Pancreatitis

(A) Changes in serum levels of amylase and lipase in C57BL6 mice treated with or without antibiotics. Mice administered with AMP (1 g/l), neomycin (NEO, 1 g/l), vancomycin (VCM, 0.5 g/l) and metronidazole (MNZ, 1 g/l) in the drinking water for 3 weeks were challenged with a high dose of cerulein (50 μg/kg i.p.) for a total of seven times; results are expressed as means ± SD. **$p < 0.01$, as compared with group not administered antibiotics.

(B) Histopathology of mice treated with a broad range of antibiotics followed by systemic injection of a high dose of cerulein (50 μg/kg i.p.) for a total of seven times. Pancreatic tissue was obtained from mice at 8 hr after the first injection of cerulein. Lack of pancreatitis in mice treated with antibiotics, bottom panel. Magnification ×200.

(C) Changes in serum levels of amylase in *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, or *Nod1*$^{-/-}$ mice without antibiotics. Mice were challenged with a high dose of cerulein (50 μg/kg i.p.) for a total of seven times. Results are expressed as means ± SD. *$p < 0.05$, **$p < 0.01$, as compared with C57BL6 WT mice.

administered FK156 before, at the same time, or after cerulein administration (Figure S2A). Thus, activation of NOD1 causes severe pancreatitis in mice treated with a low subinflammatory dose of cerulein, and thus combined low-dose cerulein and NOD1 ligand could be used as a model to test the role of NOD1 in cerulein-induced pancreatitis.

We sought to show that NOD1-ligand administration in the above-described low-dose cerulein model is acting in place of commensal bacteria. In these studies, C57BL6 mice administered drinking water containing AMP and KM were given three hourly injections of low doses of cerulein alone, an i.p. injection of ECLACZ alone, or an i.p. injection of ECLACZ and low doses of cerulein (Figure 2E). Treatment with i.p. injection of ECLACZ and low-dose cerulein resulted in acute pancreatitis as well as increased serum amounts of amylase, whereas treatment with ECLACZ or cerulein alone did not cause significant changes in these parameters. In additional studies, *Nod1*$^{-/-}$ mice were more resistant to pancreatitis induced by ECLACZ and low-dose cerulein than were WT mice and *Tlr4*$^{-/-}$ mice (Figures 2E and 2F). Thus, intestinal bacteria evoke a NOD1 response similar to that obtained with NOD1 ligand, and therefore the latter is acting as a mimic of intestinal bacteria in this setting.

NOD1 Ligand and Low-Dose Cerulein Are Synergistic Inducers of MCP-1

Prior work has shown that MCP-1 and interleukin-6 (IL-6) are associated with the local inflammation of the pancreas (Grady et al., 1997). We therefore determined the involvement of these proinflammatory mediators in the development of pancreatitis induced by FK156 in combination with low-dose cerulein. Consistent with previous reports (Fritz et al., 2007; Watanabe et al., 2010), systemic injection of FK156, but not MDP, induced increased amounts of serum MCP-1 and IP-10 chemokines. Moreover, MCP-1 induction, but not IP-10 induction, was greatly enhanced by coadministration of cerulein, even though low-dose cerulein treatment alone did not induce MCP-1 (Figure 3A).

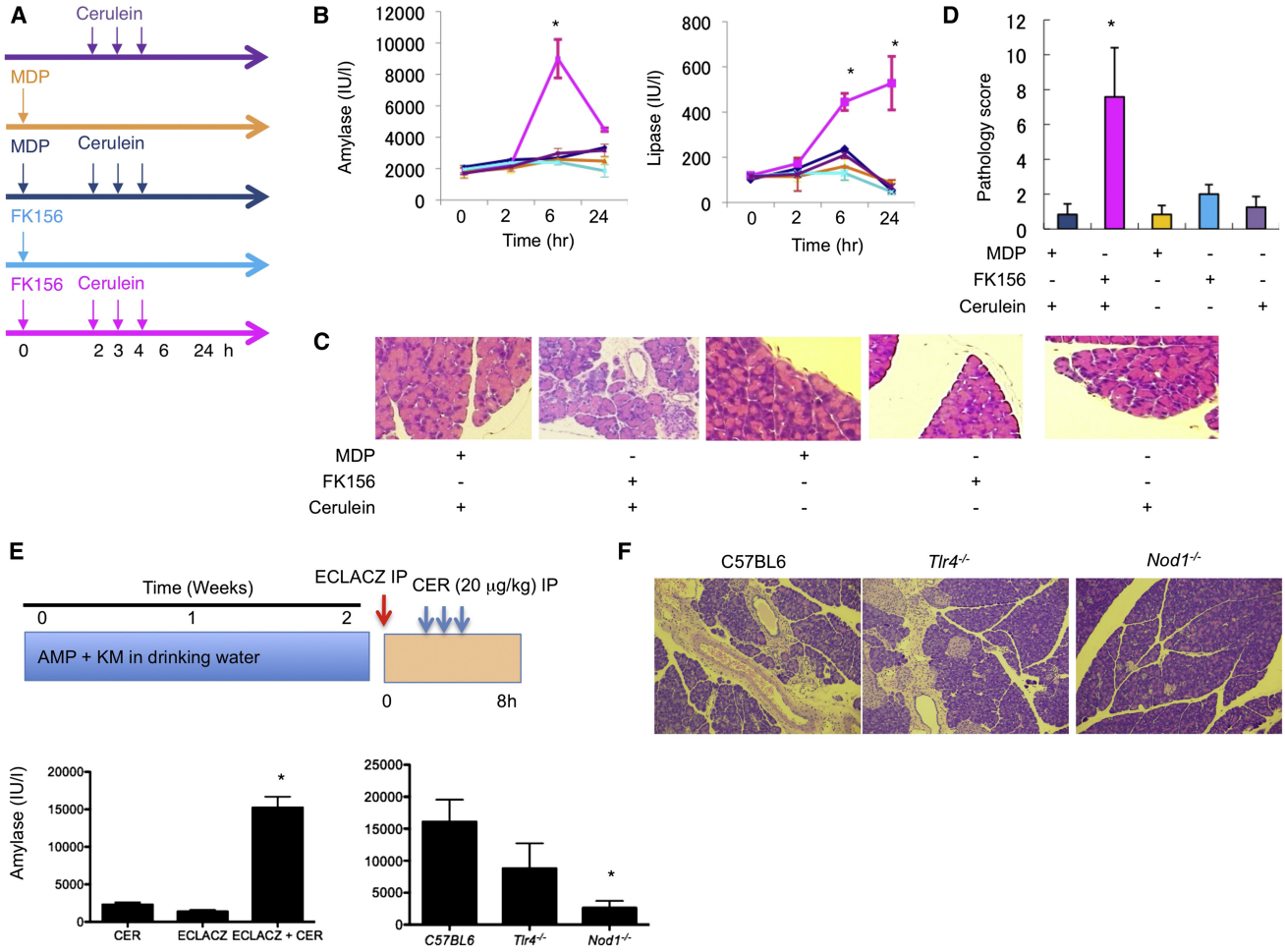


Figure 2. Induction of Acute Pancreatitis in Mice Treated with NOD1 Ligand or ECLACZ in Combination with Cerulein

(A–D) Induction of acute pancreatitis in mice treated with NOD1 ligand and cerulein.

(A) Experimental protocol: C57BL6 mice were administered FK156 (NOD1 ligand; 200 μg/mice i.p.) or MDP (NOD2 ligand; 200 μg/mice) followed by a low dose of cerulein (20 μg/kg i.p.) for a total of three times.

(B) Changes in serum levels of amylase and lipase in mice treated with NOD ligands and/or cerulein. Each color corresponds to group shown in Figure 2A. Results are expressed as means ± SD. *$p < 0.01$, as compared with other groups.

(C and D) Hematoxylin and eosin (H&E) staining (C) and histological scores (D) of pancreatic tissue harvested at 24 hr after the start of experiments; magnification ×200; each color corresponds to group shown in Figure 2A; scores are expressed as means ± SD. *$p < 0.01$, as compared with other groups.

(E and F) Induction of acute pancreatitis in mice treated with ECLACZ and cerulein.

(E) Experimental protocol. C57BL6 mice and *Tlr4*$^{-/-}$ or *Nod1*$^{-/-}$ mice exposed to AMP and KM in the drinking water were administered ECLACZ (1×10^6 cfu, i.p.) followed by three hourly injections of low doses of cerulein (CER). Changes in serum levels of amylase in C57BL6 mice treated with ECLACZ and/or cerulein, (E), left panel. Changes in serum levels of amylase in C57BL6 mice and *Tlr4*$^{-/-}$ or *Nod1*$^{-/-}$ mice treated with ECLACZ and cerulein, (E), right panel. Results are expressed as means ± SD. *$p < 0.01$, as compared with other groups. Serum levels of amylase were determined 8 hr after the treatment with ECLACZ.

(F) Lack of pancreatitis in *Nod1*$^{-/-}$ mice treated with ECLACZ and cerulein. Magnification ×200.

FK156 treatment also led to increased serum IL-6 amounts that were again enhanced by low-dose cerulein, particularly at later time points. In contrast, induction of IL-12p40 was observed either with FK156 or MDP administration, and neither response was enhanced by low-dose cerulein. Comparable increases in serum amounts of MCP-1 were observed when FK156 was administered at the time of or after cerulein administration (Figure S2A). These data indicate that the severe pancreatitis induced by treatment with NOD1 ligand and low-dose cerulein is associated with the production of MCP-1 and IL-6. Finally, similar effects on MCP-1 could be elicited by administration of ECLACZ, in that i.p. administration of the latter followed by treatment with low doses of cerulein also led to a marked increase of the serum level of MCP-1 (Figure 3B). In contrast, *Nod1*$^{-/-}$ mice administered ECLACZ and low doses of cerulein exhibited little, if any, increase in the serum level of MCP-1 (Figure 3B).

Previous studies have shown that the interaction between MCP-1 and its receptor, CCR2, plays a role in macrophage recruitment and the macrophage-dependent inflammatory response in the development of pancreatitis (Grady et al., 1997; Bhatia et al., 2005; Zhao et al., 2005). One might therefore

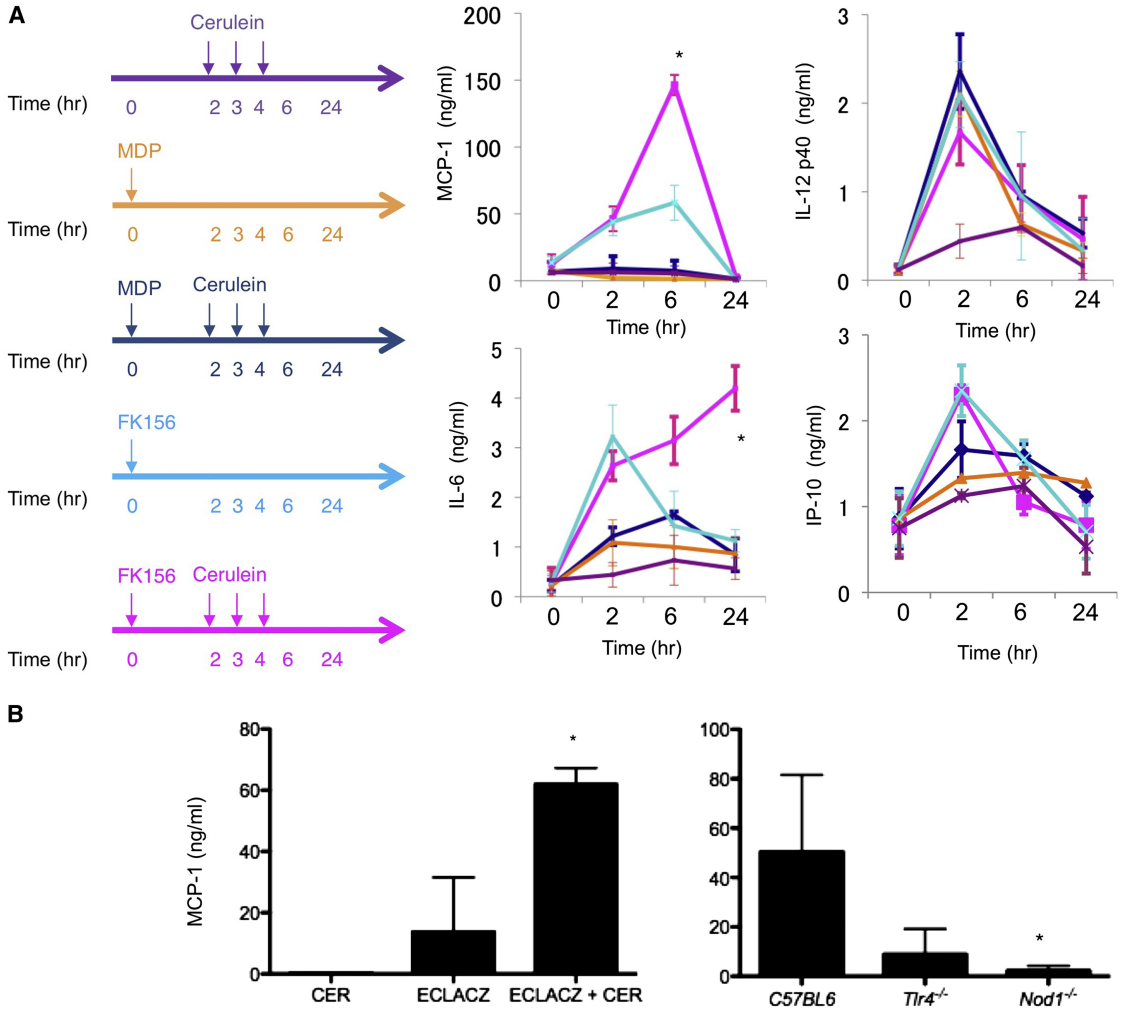


Figure 3. Concomitant Administration of NOD1 Ligand and Low-Dose Cerulein Induces Synergistic Production of MCP-1

(A) Changes in serum levels of proinflammatory cytokines and chemokines. C57BL6 mice received systemic administration of FK156 or MDP followed by i.p. injection of cerulein for a total of three times. Results are expressed as means ± SD. *$p < 0.01$, as compared with other groups. The results shown are representative of one of three experiments.

(B) Serum levels of MCP-1 in mice treated with ECLACZ and cerulein. Changes in serum levels of MCP-1 in C57BL6 mice treated with ECLACZ and/or cerulein, (B), left panel. Changes in serum levels of MCP-1 in C57BL6 mice and *Tlr4*$^{-/-}$ or *Nod1*$^{-/-}$ mice treated with ECLACZ and cerulein, (B), right panel. Results are expressed as means ± SD. *$p < 0.01$, as compared with other groups. C57BL6 mice and *Tlr4*$^{-/-}$ or *Nod1*$^{-/-}$ mice treated with AMP and KM in the drinking water were challenged with i.p. injection of ECLACZ followed by three hourly injections of low doses of cerulein. Serum levels of MCP-1 were determined 8 hr after the treatment with ECLACZ.

predict that low-dose cerulein pancreatitis is mediated by cells susceptible to MCP-1 chemoattraction. In tissue-staining studies (Figure 4A), combined administration of FK156 and low-dose cerulein was associated with massive infiltration of CD11b^{+} myeloid cells and cells expressing CCR2. In contrast, these cells did not express CXCR3, the receptor of IP-10, indicating the induction of IP-10 by NOD1 probably has little or no role in this type of inflammation.

The relationship between MCP-1 induction and the infiltration of the pancreas by CCR2^{+} cells was further explored through studies of induction of pancreatitis with FK156 and low-dose cerulein in CCR2-deficient (*Ccr2*$^{-/-}$) mice. Systemic administration of FK156 and low-dose cerulein to *Ccr2*$^{-/-}$ mice was associated with a marked reduction in the serum amylase level and pathology score as compared with WT mice, even though CCR2-deficient mice were still capable of producing MCP-1 (Figure 4B). In addition, migration of both CD11b^{+} cells and CCR2^{+} cells into the pancreas was absent in *Ccr2*$^{-/-}$ mice (Figure 4C). These studies thus showed that induction of MCP-1 by FK156 and low-dose cerulein was associated with the infiltration of inflammatory CCR2^{+} cells into the inflamed pancreas.

Low-Dose Cerulein Pancreatitis Does Not Depend on T Cells or B Cells

NOD1 is a component of the innate immune system that probably involves mainly non-T cells and non-B cells. To determine if this was in fact the case, we administered FK156 and low-dose cerulein to *Prkdc*^{scid} mice with severe combined

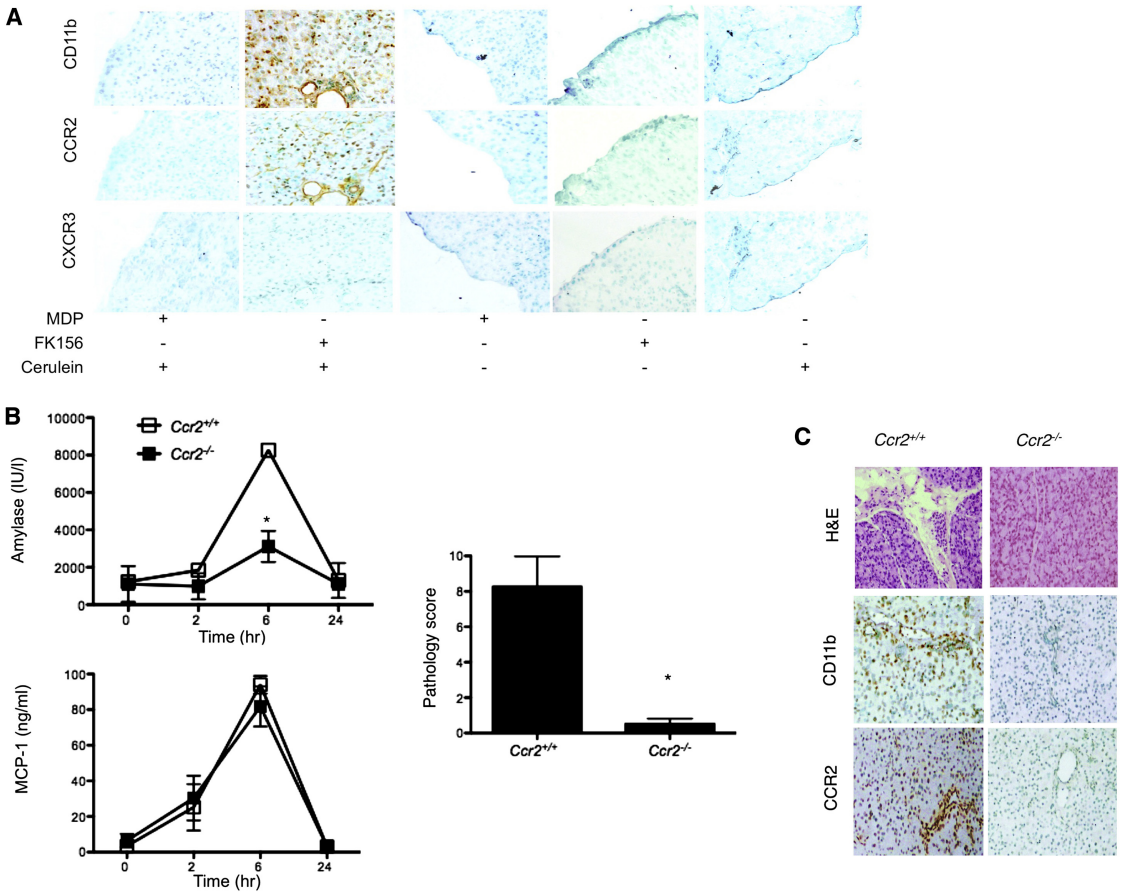


Figure 4. Migration of CCR2$^+$ Myeloid Cells Is Necessary for the Development of Pancreatitis

(A) At 6 hr after the injection of MDP or FK156, pancreatic tissue from C57BL6 mice was removed and subjected to immunohistochemical analysis for visualization of cells positive for CD11b, CCR2, and CXCR3.

(B and C) CCR2-intact (*Ccr2*$^{+/+}$) or CCR2-deficient (*Ccr2*$^{-/-}$) mice were administered FK156 followed by repeated administration of cerulein according to the protocol described in Figure 2A.

(B) Changes in serum levels of amylase and MCP-1 at the indicated time points and pathology scores of the pancreatic tissue at 24 hr; results are expressed as means ± SD. *$p < 0.01$, as compared with *Ccr2*$^{+/+}$ mice; results shown are representative of one of three experiments.

(C) Pancreatic tissue was removed at 6 hr after the start of the experiments and subjected to immunohistochemical analysis for visualization of cells positive for CD11b and CCR2; magnification ×200.

immunodeficiency (SCID) that lacked both T cells and B cells. This treatment induced equally increased amounts of serum amylase and MCP-1 in WT and SCID mice (Figure S2B), suggesting that the acute pancreatitis induced by systemic injection of NOD1 ligand and low-dose cerulein does not depend on T cells or B cells.

Acute Pancreatitis Requires Activation of NOD1 in Nonhematopoietic Cells

We next turned our attention to the cellular location of the NOD1 involved in the acute pancreatitis model studied above. Our initial approach to this question was to conduct bone marrow (BM) chimera studies to determine if NOD1 was acting in hematopoietic cells or nonhematopoietic cells for the development of pancreatitis, but first we had to define the responses of *Nod1*$^{-/-}$ cells to be used in such studies. To this end, we subjected *Nod1*$^{-/-}$ mice and *Nod1*$^{+/+}$ mice to treatment with FK156 or MDP and low-dose cerulein. *Nod1*$^{+/+}$ mice, but not *Nod1*$^{-/-}$ mice, developed pancreatitis after such FK156 and cerulein administration, as evidenced by serum amylase and MCP-1 elevations and histology scores (Figure 5A). These studies indicated that NOD1 stimulation is essential for the induction of FK156 and low-dose cerulein pancreatitis.

We then performed the BM chimera studies mentioned above and showed that administration of FK156 and low-dose cerulein led to pancreatitis in *Nod1*$^{+/+}$ green fluorescent protein (GFP)-transgenic mice reconstituted with *Nod1*$^{-/-}$ BM cells (Figure 5B), but not in *Nod1*$^{-/-}$ mice reconstituted with *Nod1*$^{+/+}$ BM cells. Thus, the development of FK156 and cerulein acute pancreatitis involves NOD1 signaling in nonhematopoietic cells.

In a second approach to the identification of the site of NOD1 activity in this model, we conducted in vitro studies of isolated pancreatic acinar cells from untreated mice to determine whether these cells exhibited NOD1 activity following stimulation with FK156 and/or low-dose cerulein (Figure S3A). Although stimulation of acinar cells with FK156 alone induced a substantial

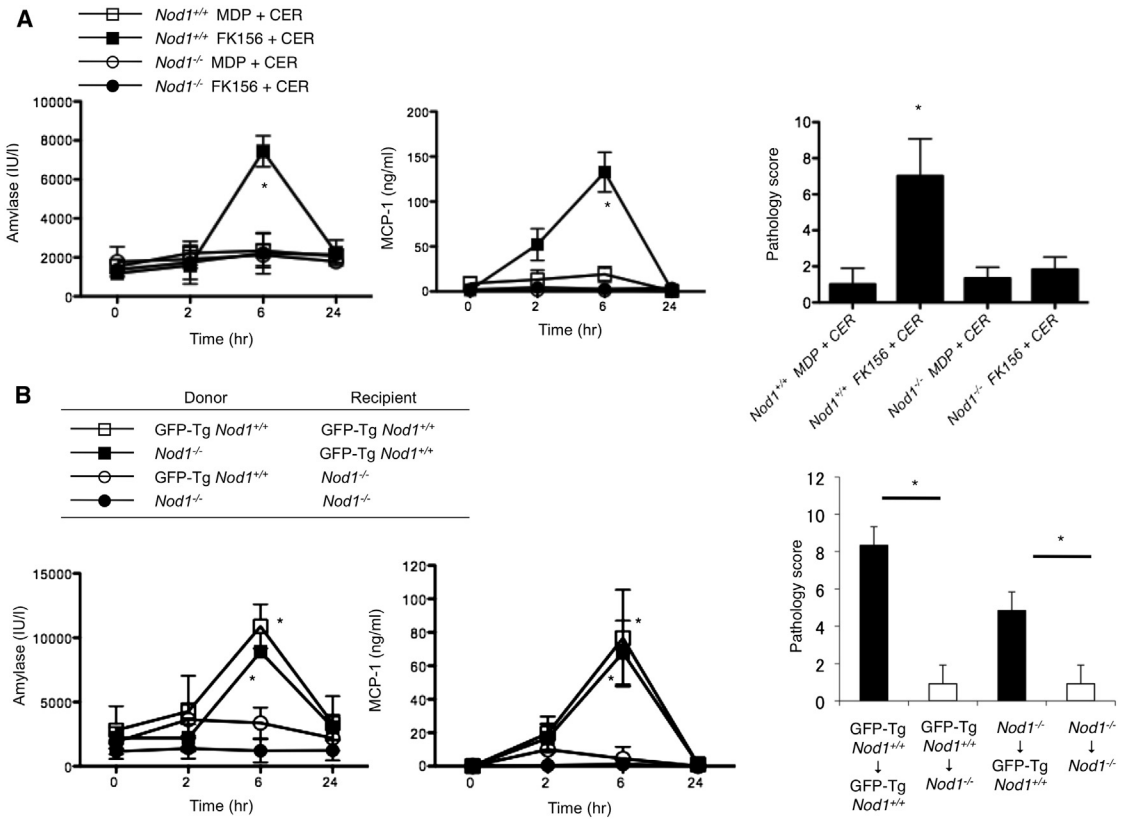


Figure 5. The Importance of NOD1 Signaling in Nonhematopoietic Cells for the Development of Severe Acute Pancreatitis

(A) *Nod1*$^{+/+}$ or *Nod1*$^{-/-}$ mice were treated with NOD ligands (FK156 or MDP) and/or cerulein (CER) as described in Figure 2A. Changes in serum levels of amylase and MCP-1 at the indicated time points, and pancreatitis histology score at 24 hr. *$p < 0.01$, as compared with the other groups.

(B) BM cells from GFP-transgenic (GFP-Tg) mice or *Nod1*$^{-/-}$ mice were transplanted into irradiated GFP-transgenic mice or *Nod1*$^{-/-}$ mice. These chimeric mice were treated with FK156 followed by repeated administration of CER. Changes in serum levels of amylase and MCP-1 at the indicated time points, and pancreatitis histology score at 24 hr. Results are expressed as means ± SD. *$p < 0.01$, as compared with the other groups. *$p < 0.01$ (pathology score). The results shown are representative of one of three experiments.

amount of MCP-1, synergistic production of MCP-1 was seen upon stimulation with FK156 plus low-dose cerulein in an FK156-dose-dependent fashion. In contrast, FK156 alone was sufficient for the induction of IP-10 production. Finally, the induction of MCP-1 under these conditions required the presence of NOD1 (Figure S3B). These studies established, first, that pancreatic acinar cells are the nonhematopoietic cellular site of NOD1 activation in the FK156 and low-dose cerulein model of acute pancreatitis. Second, they showed that MCP-1 induction requires dual NOD1 and low-dose cerulein signaling via NOD1 and CCKR, respectively.

As mentioned in Figure 3A, administration of FK156 and low-dose cerulein elicited elevations in circulating amounts of IL-6 as well as MCP-1. This finding correlates with the fact that acinar cells from *Nod1*$^{+/+}$ mice, but not cells from *Nod1*$^{-/-}$ mice, produced IL-6 upon stimulation with FK156 and low-dose cerulein and suggests that, as in the case of MCP-1, increased circulating amounts of IL-6 originate from acinar cells (Figure S3B). It seemed probable that the MCP-1 and IL-6 secretion might be interrelated, given that IL-6 participates in MCP-1 production via STAT3 activation (Deshmane et al., 2009). To address this possibility, we measured acinar cell production of MCP-1 in cells isolated from *Nod1*$^{+/+}$ mice and then treated with FK156 and low-dose cerulein in the presence or absence of neutralizing IL-6 receptor (IL-6R) antibody (Ab). This blockade of IL-6 signaling inhibited the production of MCP-1, but only partially (Figure S3C); thus, IL-6 is not the only inducer of MCP-1 in this model.

NOD1 Plus Low-Dose Cerulein Signaling Induces Transactivation of STAT3 and NF-κB In Vivo

Because acinar cell MCP-1 production is a key factor in the development of FK156 and low-dose cerulein pancreatitis, we next turned our attention to the signaling pathways controlling such production. Previous studies have shown that MCP-1 production depends on STAT3 and NF-κB activation (Deshmane et al., 2009), and, indeed, we verified that these factors were necessary for the induction of FK156 and low-dose cerulein pancreatitis by measuring activation of both STAT3 and NF-κB in the pancreatic tissue of mice with pancreatitis. Nuclear expression of phospho-STAT3 (pSTAT3) and phospho-IκBα (pIκBα) was seen in the pancreatic acinar cells expressing amylase of *Nod1*$^{+/+}$ mice treated with low-dose cerulein and FK156, whereas little or no pSTAT3 or pIκBα was seen in acinar

cells of *Nod1*$^{-/-}$ mice (Figure S3D). Similar findings were obtained using conventional immunohistochemical analysis (Figure S3E).

To confirm the above findings, we performed staining studies of tissue obtained from BM-chimeric mice, consisting of irradiated *Nod1*$^{+/+}$ mice bearing a GFP-expressing transgene and irradiated *Nod1*$^{-/-}$ mice, reconstituted with *Nod1*$^{-/-}$ GFP-transgene-negative BM cells and *Nod1*$^{+/+}$ GFP-transgene-positive BM cells, respectively. Replacement of BM cells was confirmed by flow-cytometric analysis of GFP expression at 8 weeks after the transplantation (Figure S4A). We found that amylase-expressing acinar cells were GFP-positive in irradiated GFP-transgene-positive mice reconstituted with GFP-transgene-negative *Nod1*$^{-/-}$ BM cells, whereas they were GFP-negative in irradiated GFP-transgene-negative *Nod1*$^{-/-}$ mice reconstituted with GFP-transgene-positive *Nod1*$^{+/+}$ BM cells (Figure S4B). Thus, the GFP status of the acinar cells reflected the GFP status of the recipient, not the donor. In additional studies, we showed that expression of pIκBα and pSTAT3 was seen in acinar cell nuclei of irradiated GFP-transgene-positive *Nod1*$^{+/+}$ mice reconstituted with *Nod1*$^{-/-}$ BM cells (Figure S4B). In contrast, expression of these factors was barely detectable in acinar cell nuclei of irradiated GFP-transgene-negative *Nod1*$^{-/-}$ mice reconstituted with GFP-transgene positive *Nod1*$^{+/+}$ BM cells (Figure S4B). Similar findings were obtained using conventional immunohistochemical analysis (Figure S4C). These data thus provide additional evidence that activation of STAT3 and NF-κB in response to FK156 and low-dose cerulein administration is occurring in pancreatic acinar cells.

Administration of JSI-124, a specific STAT3 inhibitor, inhibited nuclear translocation of STAT3, and administration of IMD-0354, an NF-κB inhibitor, inhibited nuclear translocation of the NF-κB subunit p65 in the pancreas of mice administered FK156 and low-dose cerulein, as assayed in the Transfactor Binding Assay, establishing that these agents do in fact exert the expected inhibition of activation of STAT3 and NF-κB under these conditions (Figure S5A). In addition, administration of JSI-124 or IMD-0354 alone led to a partial reduction of serum levels of amylase and MCP-1, whereas administration of both agents led to complete reduction of serum levels of amylase and MCP-1 (Figure S5B). A similar result was obtained in respect to the inflammation score of the pancreas (Figure S5C). These studies, using pharmacological inhibitors for STAT3 and NF-κB, thus provide evidence that activation of both STAT3 and NF-κB in acinar cells acts in an additive fashion to bring about the development of pancreatitis induced by FK156 and low-dose cerulein.

Molecular Mechanisms Underlying the Activation of STAT3 and NF-κB in FK156 and Low-Dose Cerulein Pancreatitis

Having established that STAT3 and NF-κB are critical signaling components in the induction of FK156 and low-dose cerulein pancreatitis, we were interested in establishing how FK156 and low-dose cerulein act together to induce these components, whereas neither stimulant do so alone. In initial studies we focused on the signaling pathways leading to STAT3 activation and showed that treatment with FK156 alone induced expression of pSTAT3 in the pancreas of *Nod1*$^{+/+}$ mice, but not *Nod1*$^{-/-}$ mice (Figure 6A). This is consistent with our previous report that NOD1 ligand induces type I IFN production (Watanabe et al., 2010) as well as with a study showing that type I IFN activates various STATs, including STAT3 (Yang et al., 1998). Similarly, treatment with cerulein alone induced pSTAT3 expression (in both *Nod1*$^{+/+}$ and *Nod1*$^{-/-}$ mice), presumably as a result of direct CCKR activation of JAK2 (Ferrand et al., 2005). However, treatment of *Nod1*$^{+/+}$ mice (but not *Nod1*$^{-/-}$ mice) with both FK156 and low-dose cerulein induced higher pSTAT3 expression as compared with either stimulus alone, and this was associated with similarly increased nuclear translocation (Figure 6B). Thus, FK156 and low-dose cerulein reinforce one another in STAT3 activation.

We also noted that expression of pSTAT1 was induced in the pancreas of *Nod1*$^{+/+}$ mice treated with FK156, but, in this case, such expression was not enhanced by combined treatment with FK156 and cerulein (Figure 6A). Moreover, expression of pSTAT1 was not induced in the pancreas of *Nod1*$^{-/-}$ mice, whether mice were treated with FK156 or cerulein. These results were corroborated by measurement of nuclear translocation (Figure 6B). Given the fact that STAT1 is a critical downstream signaling molecule for type I IFN, these data suggest that FK156 also activates a type I IFN pathway, as reported previously (Watanabe et al., 2010).

We next examined the signaling pathways leading to NF-κB activation. Although previous studies utilizing human embryonic kidney cells showed rapid activation of NF-κB upon stimulation with NOD1 ligands (Chamaillard et al., 2003; Fritz et al., 2007), the roles of NF-κB in NOD1-mediated signaling pathways have been poorly defined in primary pancreatic acinar cells. In initial studies, we found that neither NOD1 ligand nor low-dose cerulein administration alone resulted in expression of pIκBα or degradation of IκBα (Figure 6A). This is consistent with our previous report that NOD1 stimulation alone is a poor inducer of NF-κB in intestinal epithelial cells (Watanabe et al., 2010) as well as previous reports by other investigators that cerulein is also a poor inducer of NF-κB at low concentrations (Yu et al., 2005). Nevertheless, combined stimulation did result in such induction as shown by expression of pIκBα and nuclear translocation of p65 and p50 (Figures 6A and 6B). In explanation of this finding, we focused first on CCK signaling and noted that previous studies had shown that CCKR signaling induces protein kinase C (PKC) activation (Rakonczay et al., 2008; Tando et al., 1999). To verify that this pathway is in play in the low-dose cerulein model we treated mice with low-dose cerulein (in the presence or absence of FK156) and showed that such mice exhibited enhanced pancreatic cell expression of phospho-PKC (pPKC) (Figure 6A). Because both activated PKC generated by cerulein signaling and RICK (receptor-interacting protein-like interacting caspase-like apoptosis regulatory protein kinase) generated by NOD1 signaling are known to activate NF-κB via phosphorylation and/or ubiquitination of transforming growth factor β (TGF-β)-activated kinase 1 (TAK1) (Hasegawa et al., 2008; Shinohara et al., 2005), we next considered the possibility that TAK1 is a signaling molecule that serves as a nodal point in the low-dose cerulein and FK156 signaling pathways that facilitate NF-κB activation. In studies to explore this possibility, we performed immunoprecipitation studies of cell lysates isolated from the pancreas of *Nod1*$^{+/+}$ and *Nod1*$^{-/-}$ mice. We found that treatment with cerulein, but not FK156, induced physical interaction between

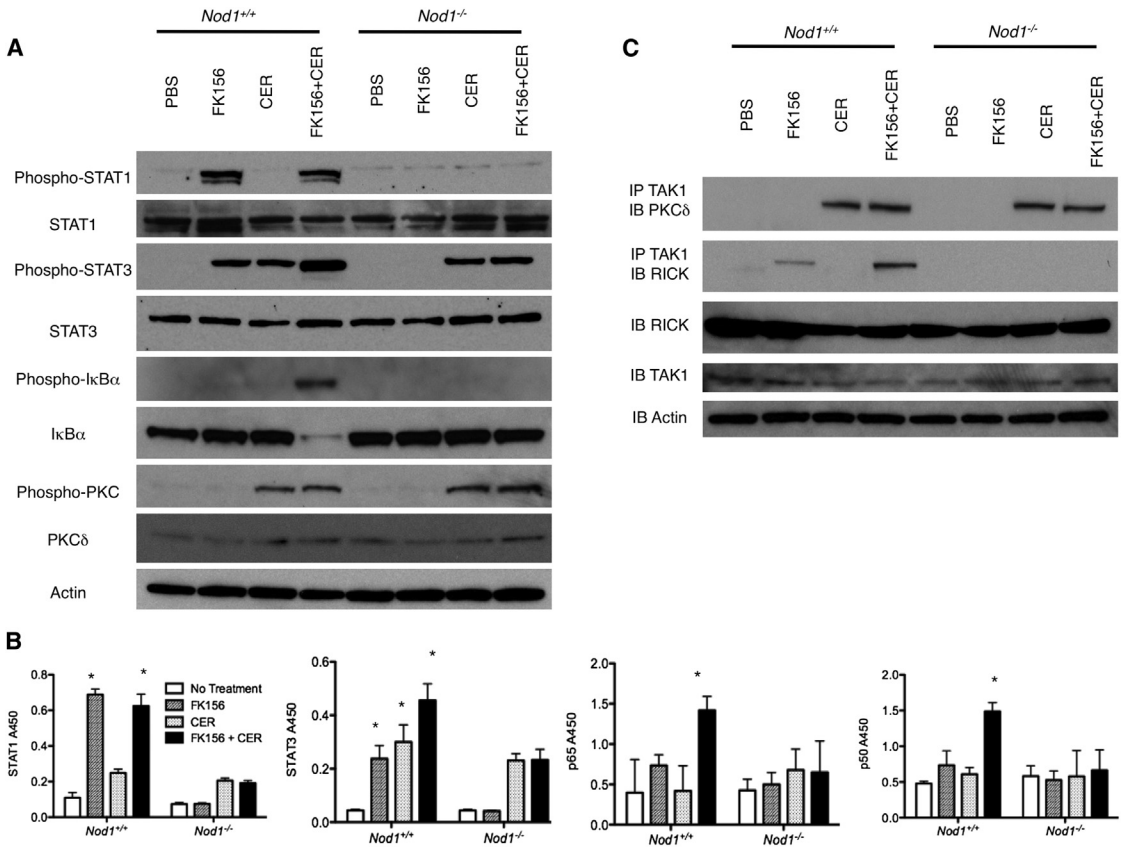


Figure 6. NOD1 Activation Followed by Cerulein Challenge Induces Transactivation of STAT3 and NF-κB In Vivo

Nod1$^{+/+}$ or *Nod1*$^{-/-}$ mice were treated with FK156 and/or cerulein (CER) as described in Figure 2A. Whole or nuclear extracts were prepared from the pancreas of mice at 5 hr after systemic challenge with FK156 and subjected to immunoblotting.

(A) The expression of pSTAT3, STAT3, pSTAT1, STAT1, pPKC, PKCδ, pIκBα, IκBα, and actin was shown with the use of whole-pancreatic extract.

(B) Activation of STAT1, STAT3, p65, and p50 with the use of nuclear extracts, as assessed by Transfactor assay. Results are expressed as means ± SD. *$p < 0.01$, as compared with the untreated group. The results shown are representative of one of three experiments.

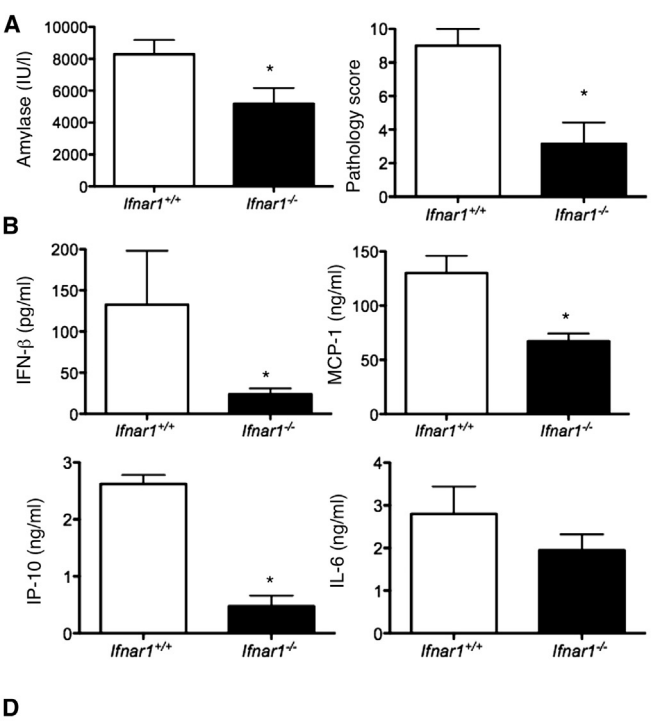
(C) Physical interaction between RICK and TAK1 or between PKCδ and TAK1 in whole-pancreatic extracts. Pancreatic extracts were immunoprecipitated (IP) with TAK1 Ab, followed by immunoblotting (IB) with PKCδ or RICK Ab.

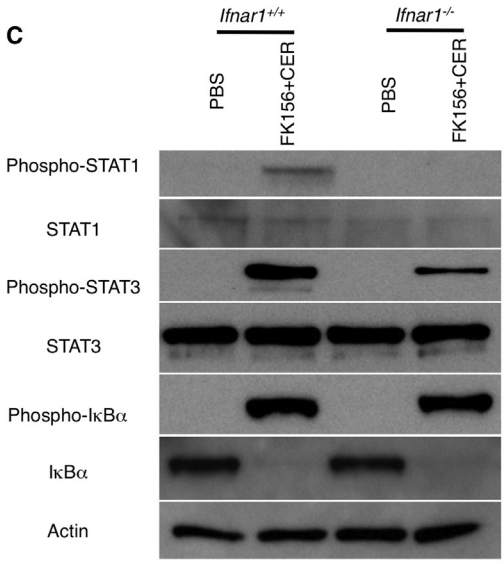
TAK1 and PKCδ in pancreatic cells of *Nod1*$^{+/+}$ and *Nod1*$^{-/-}$ mice (Figure 6C), suggesting induction of TAK1 activation through cerulein-mediated PKC activation. More importantly, as shown by immunoprecipitation with TAK1 Ab followed by immunoblotting with RICK Ab, interaction between RICK and TAK1 was markedly increased in the pancreas of mice treated with both FK156 and low-dose cerulein as compared to the marginal interaction observed with FK156 alone (Figure 6C). Taken together, these data are consistent with the hypothesis that synergistic NF-κB activation by low-dose cerulein and NOD1 is due to cerulein-induced PKC-TAK1 activation followed by opportunistic RICK utilization of the activated TAK1 to induce NF-κB.

NOD1-Induced Activation of STAT3 via Type I IFN

Finally, to more fully assess the role of NOD1 signaling in STAT3 activation via type I IFN, we investigated FK156 and low-dose cerulein induction of pancreatitis in IFN-αβ receptor (IFN-αβR)-deficient (*Ifnar1*$^{-/-}$) mice. In initial studies, we showed that *Ifnar1*$^{-/-}$ mice exhibited an attenuated pancreatitis along with a significant reduction of serum levels of amylase and MCP-1 as compared with those in *Ifnar1*$^{+/+}$ mice (Figures 7A and 7B). In addition, upon challenge with FK156 and low-dose cerulein, pancreatic expression of pSTAT3 in *Ifnar1*$^{-/-}$ mice was significantly reduced as compared to that in *Ifnar1*$^{+/+}$ mice, suggesting that type I IFN signaling is necessary for maximal STAT3 activation in this model (Figure 7C).

As expected, pancreatic expression of pSTAT1 was completely absent in *Ifnar1*$^{-/-}$ mice upon challenge with FK156 and low-dose cerulein, suggesting that pancreatic STAT1 activation is dependent upon NOD1-mediated type I IFN signaling in this model. In addition, consistent with the results of the signaling studies presented above, no significant difference in NF-κB activation was seen between *Ifnar1*$^{-/-}$ and *Ifnar1*$^{+/+}$ mice, as assessed by pancreatic expression of pIκBα and IκBα. Serum levels of IFN-β and IP-10, whose production requires activation of type I IFN signaling, were markedly reduced in *Ifnar1*$^{-/-}$ mice treated with FK156 and low-dose cerulein as compared with those in *Ifnar1*$^{+/+}$ mice. In contrast, no significant difference was seen in production of IL-6, whose production





D

Peritoneal Lavage				Blood			
PBS (n=7)	FK156 (n=7)	CER (n=7)	FK156+CER (n=7)	PBS (n=7)	FK156 (n=7)	CER (n=7)	FK156+CER (n=7)
0 (0)	0 (0)	14.2 (1)	42.8 (3)	0 (0)	0 (0)	71.4 (5)	85.7 (6)

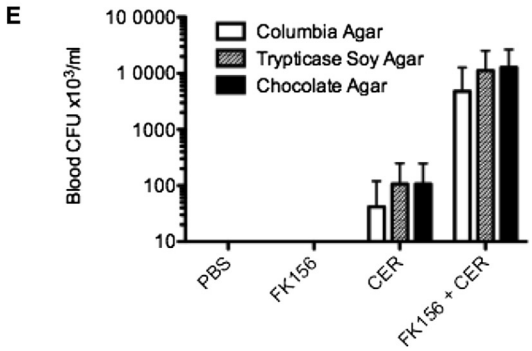


Figure 7. Involvement of Type I IFN Signaling in the Development of Pancreatitis

(A–C) IFNαβ receptor-intact (*Ifnar1*$^{+/+}$) or -deficient (*Ifnar1*$^{-/-}$) mice were subjected to induction of FK156 and low-dose cerulein pancreatitis, as indicated in Figure 2.

(A and B) Serum levels of amylase (A) and MCP-1, IL-6, IFN-β, and IP-10 (B) at 6 hr after systemic challenge with FK156 and pathology scores (A) of the pancreatic tissue at 24 hr after systemic challenge with FK156. Results are expressed as means ± SD. *$p < 0.01$, as compared with *Ifnar1*$^{+/+}$ mice.

(C) Whole-cell extracts were prepared from pancreatic tissue of mice at 5 hr after systemic challenge with FK156 and subjected to immunoblotting to determine expression of pSTAT3, STAT3, pSTAT1, STAT1, pIκBα, IκBα, and actin. The results shown are representative of one of two experiments.

(D and E) C57BL6 mice were subjected to induction of FK156 and low-dose cerulein pancreatitis; blood and peritoneal lavage fluid were collected 8 hr after the injection.

(D) Percentage and number (parentheses) of mice that were positive for bacterial culture in the blood and peritoneal lavage fluids.

(E) Bacterial loads in the blood determined by counting colonies in columbia agar, trypticase soy agar, and chocolate agar plates. Results are expressed as means ± SD and a summary of three independent experiments.

depends mainly upon activation of NF-κB. Taken together, these results indicate that type I IFN signaling induced by NOD1 activation is involved in the development of pancreatitis in this model.

Bacterial Translocation in Low-Dose Cerulein-Induced Pancreatitis

Intestinal barrier dysfunction and translocation of intestinal microflora into the systemic circulation play a critical role in the development of severe acute pancreatitis (Frossard et al., 2008; Rychter et al., 2009). We therefore addressed the role of bacterial translocation with studies of bacteremia in mice treated with low-dose cerulein alone or in combination with FK156. We found that 5 of 7 mice treated with low-dose cerulein alone exhibited bacteremia, but that the level of bacteremia was relatively low in each case (Figures 7D and 7E). On the other hand, 6 of 7 mice subjected to combined treatment with FK156 and low-dose cerulein exhibited bacteremia, and in this case, the level of bacteria was much higher than that obtained by treatment with low-dose cerulein alone.

To more clearly identify the role of bacterial translocation from the gut into the circulation in low-dose cerulein pancreatitis, we treated mice with drinking water containing AMP and KM and administered oral ECLACZ prior to FK156 and low-dose cerulein administration. We again observed bacteremia, in this case at a level that increased in parallel with the oral ECLACZ dose

(Figure S6). As expected, the peak level of bacteremia was not as high as that obtained with normal "unperturbed" microflora in the initial study, probably due to the relative paucity of organisms present in the gut under these circumstances. However, it should be noted that serum amylase levels increased in proportion to the oral ECLACZ dose, indicating that even these relatively low levels of bacteremia supported the development of pancreatitis in mice that were administered FK156 and low-dose cerulein (Figure S6). These data are consistent with the view that low-dose cerulein administration alone does not induce pancreatitis in the absence of NOD1 ligand and, in addition, does not induce sufficient bacterial translocation to facilitate de novo NOD1 activation due to exposure to low levels of translocated organisms. In contrast, FK156 and low-dose cerulein administration not only initiated pancreatitis, but also caused bacterial translocation that can now sustain the pancreatitis.

DISCUSSION

We explored the mechanism by which commensal intestinal bacteria contribute to the development of pancreatitis induced by cerulein, a CCKR agonist. Our initial studies established that high-dose cerulein administration, capable of causing pancreatitis by itself, required the presence of a normal intestinal bacterial flora and, quite surprisingly, acted via stimulation of NOD1, an intracellular sensor of a peptide derived from the bacterial wall of both gram-positive and -negative bacteria. To further study this phenomenon, we developed a model of pancreatitis in which mice were administered a dose of cerulein that by itself was insufficient to cause pancreatitis but was nevertheless able to cause pancreatitis when coadministered with NOD1 ligand. This model thus allowed us to identify the specific roles of cerulein and NOD1 signaling in the induction of pancreatic inflammation.

Two key immunopathologic features of the pancreatitis in the low-dose cerulein model are that the inflammation is associated with acinar cells that produce large amounts of MCP-1, a chemoattractant of $CCR2^+$ cells, and that $Ccr2^{-/-}$ mice are almost completely resistant to the development of pancreatitis in this model. These findings strongly suggest that the pancreatitis is driven by MCP-1-mediated migration of $CCR2^+$ inflammatory cells into the pancreas. This conclusion is consistent with previous studies showing that abrogation of MCP-1:CCR2 interactions via administration of a plasmid expressing mutated MCP-1 or a blocker of MCP-1 synthesis protects animals from experimental pancreatitis (Bhatia et al., 2005; Zhao et al., 2005). In addition, in a clinical study, Regnér et al. (2008) showed that serum levels of MCP-1 at the time of a patient's admission to hospital are strongly associated with development of severe acute pancreatitis. Thus, it appears that acinar cell production of MCP-1 and MCP-1-dependent cell migration are essential to the development of acute pancreatitis, not only in this model, but also in other models, and in human pancreatitis as well.

The importance of MCP-1 to pancreatitis accounts for the need for NOD1 signaling in the induction and maintenance of this inflammation. Previous studies have shown that activation of both NF-κB and STAT3 is required for maximal production of MCP-1 (Deshmane et al., 2009; Ray et al., 2008). In the present studies, we corroborated this finding in the context of pancreatitis by showing that both the NF-κB and STAT3 signaling pathways are activated during MCP-1 induction and, indeed, pharmacological inhibition of both pathways is required for the suppression of pancreatitis. In addition, we found that although FK156 or cerulein could induce STAT3 alone, both were necessary for optimal induction and, perhaps more importantly, although neither stimulant could induce activation of the NF-κB signaling pathway alone, they could induce this pathway when acting in concert. It follows that NOD1 signaling is essential to pancreatitis induction because it is necessary for robust activation of the signaling pathways that lead to the production of MCP-1.

The poor NF-κB activation by NOD1 signaling alone in the present study is consistent with our previous studies of this signaling pathway. We showed in these studies that NOD1 activation of RICK (the adaptor protein immediately downstream of NOD1) leads to RICK binding to TRAF3, and thus to activation of TBK1 and IRF7; this, in turn, leads to induction of IFN-β and signaling via IFNαβR for inducing IFN-stimulated gene factor 3, a complex composed of STAT1, STAT2, and IRF9 that acts as a transcription factor for chemokines including IP-10 (Watanabe et al., 2010). Thus, in effect, NOD1 signaling bypasses NF-κB to induce chemokines via a type I IFN-dominated pathway. How, then, does NOD1 signaling cause NF-κB activation in association with low-dose cerulein? We believe the answer lies in the fact that even low doses of cerulein induce activation of TAK1 via PKC (Li et al., 2009; Shinohara et al., 2005) and that in the presence of such activation, RICK activated by NOD1 forms a complex with TAK1 to activate NF-κB. This idea is supported by an enhanced physical interaction between RICK and TAK1 in the mice treated with both FK156 and cerulein.

With respect to STAT3 activation in this model, previous studies have shown that cerulein can induce STAT3, at least at suboptimal levels (Yu et al., 2005). However, as shown in these studies, NOD1 signaling greatly augments such induction by at least two mechanisms. On the one hand, by facilitating NF-κB activation, NOD1 promotes the synthesis of IL-6, a cytokine capable of STAT3 activation. On the other hand, NOD1 signaling leads to production of type I IFN, a cytokine also capable of STAT3 activation (Yang et al., 1998).

Analysis of the level of bacteremia occurring after administration of a low dose of cerulein alone or in combination with NOD1 ligand provided insight into the role of bacterial entry into the circulation in the induction of low-dose cerulein pancreatitis. Low-level bacteremia was observed in mice treated with low doses of cerulein alone, indicating that the level of activation of CCKR with this dose leads to some degree of translocation of bacteria into the systemic circulation. Evidently, however, this level of translocation does not provide a sufficient NOD1 signal in acinar cells to initiate and sustain pancreatitis. In contrast, higher levels of bacteremia accompanied administration of NOD1 ligand and low-dose cerulein. It appears that such bacteremia, by providing a continued source of NOD1 signaling, is necessary to sustain the pancreatitis initiated by exogenous NOD1 ligand. Thus, low-dose cerulein-induced pancreatitis is equivalent to high-dose cerulein pancreatitis, except for the fact that the latter initiates a level of pancreatic inflammation

that causes sufficient bacterial translocation and NOD1 signaling on its own.

It has been generally assumed that NOD1 activation is induced exclusively by pathogenic organisms and that such activation elicits NOD1-mediated host defense responses (Girardin et al., 2003; Kim et al., 2004). However, it was recently shown that NOD1 signaling can also be initiated by commensal organisms in the neonatal gut, where it plays a role in lymphoid-tissue organogenesis via the induction of defensin and the production of a chemotactic factor (Bouskra et al., 2008). The present study extends this observation by showing that NOD1 activation by commensal organisms also occurs during an inflammatory response. These observations raise the question of why NOD1 activation by commensal organisms does not cause inflammation in the normal gut. We believe the answer to this question is that NOD1 does respond to commensal organisms in the normal uninflamed gut via type I IFN signaling rather than NF-κB signaling, and type I IFN signaling does not result in inflammation (Abe et al., 2007).

In conclusion, in the present study, we provide evidence that activation of NOD1 by commensal organisms can facilitate a severe form of acute pancreatitis by acting in synergy with low doses of a CCKR agonist, cerulein. In addition, we show that such synergy is necessary for the production of MCP-1, a chemokine that mediates migration and infiltration of $CCR2^+$ pathogenic myeloid cells into the pancreas. Finally, we show that a critical feature of this model of pancreatitis, as well as high-dose cerulein pancreatitis, that does not require priming by NOD1 agonist, is that pancreatitis is driven by commensal organisms that stimulate NOD1 in acinar cells. Thus, although autodigestion of acinar cells mediated by activation of pancreatic enzymes such as trypsinogen is one necessary component of acute pancreatitis, bacterial translocation and the induction of acinar cell expression of inflammatory mediators are also necessary components (Dawra et al., 2011; Ji and Logsdon, 2011). It seems probable that therapeutic measures attacking these latter components, such as those directed at the inhibition of NOD1 signaling, offer a new approach to the treatment of acute pancreatitis.

EXPERIMENTAL PROCEDURES

Mice

C57BL6 mice, GFP-transgenic mice (Okabe et al., 1997), and *Prkdc*^{scid} mice were purchased from CLEA. *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, and *Tlr9*$^{-/-}$ mice were purchased from Oriental Bioservice. In some experiments, *Nod1*$^{-/-}$ (Watanabe et al., 2010) and *Ccr2*$^{-/-}$ mice (Boring et al., 1998) were used. Mice were reared under specific pathogen-free conditions. Animal use adhered to the Kyoto University animal-care guidelines, and protocols of animal experiments were approved by the review boards of Kyoto University.

Induction of Pancreatitis

Mice received i.p. injection of FK156 (200 μg) or MDP (200 μg) in combination with i.p. injection of cerulein (20 μg/kg) for a total of three times. Mice treated with AMP (1 g/l) and KM (1 g/l) in the drinking water for 2 weeks were challenged with i.p. injection of ECLACZ (Yoshida et al., 2001) (1×10^6 cfu) followed by i.p. injection of cerulein (20 μg/kg) for a total of three times. Mice treated with AMP (1 g/l), neomycin (1 g/l), KM (0.5 g/l), or metronidazole (1 g/l) in the drinking water for 3 weeks were challenged with i.p. injection of cerulein (50 μg/kg) for a total of seven times. Sera and pancreas tissue were obtained at the indicated time points.

Bone Marrow Transplantation

For the generation of BM-chimeric mice, recipient mice were irradiated with 10 Gy and were reconstituted with BM cells from the donor mice as previously described (Watanabe et al., 2010).

NF-κB and STAT3 Activation Assay

Whole extracts isolated from the pancreas were subjected to immunoblotting as previously described (Watanabe et al., 2010). pSTAT1, STAT3, pSTAT3, IκBα, pIκBα, pPKC, and PKCδ Abs were obtained from Cell Signaling. Actin, TAK1, and STAT1 Abs were obtained from Santa Cruz Biotechnology. Binding activity of nuclear extract to NF-κB subunits (p50 and p65), STAT1, and STAT3 was measured using a TransAM kit, obtained from Active Motif, as previously described (Watanabe et al., 2010). 7 or 15 μg of nuclear extracts were subjected to assay. Physical interaction between RICK and TAK1 or between PKCδ and TAK1 was analyzed via immunoprecipitation using whole-pancreatic extract. Pancreatic extracts were immunoprecipitated with TAK1 Ab followed by immunoblotting with PKCδ or RICK Ab (Cayman) as described previously (Watanabe et al., 2010).

Statistical Analysis

Student's t test was used to evaluate the significance of the differences. A value of $p < 0.05$ was regarded as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.05.024.

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